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Proliferative Activity of Esophageal Carcinomas and Their Lymph Node Metastases: Comparison Using Argyrophilic Nucleolar Organizer Region Staining

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Background: Many reports have concluded that quantification of the argyrophilic nucleolar organizer regions (AgNORs) measures proliferative activity and is a prognostic indicator in malignant disease. This retrospective study set out to evaluate the relationship between the AgNORs of the primary tumors and those of lymph node metastases in esophageal carcinoma.

Methods: Using a one-step silver staining technique, AgNORs were counted in surgical specimens from 54 patients with squamous cell carcinomas.

Results: The AgNOR scores of the lymph nodes metastases were significantly lower than those of the primary tumor ($P = 0.0001$). In 53 of 54 cases (98%), the AgNOR scores in the nodal metastases were lower than those of the primary tumor. The survival of 22 patients with AgNOR scores $\geqq 4.0$ for the primary tumor was significantly less than that of 32 patients with AgNOR scores <4.0 for the primary tumor ($P = 0.0014$).

Conclusions: The AgNOR score of the lymph node metastases had no prognostic significance. The AgNOR score of esophageal primary cancer reflects the prognosis of patients. Scores for lymph node metastases were lower and did not reflect prognosis. The lower score in the lymph node metastases may result from the antitumor activity of macrophages in the lymph nodes. *J. Surg. Oncol. 1997;65:274–279.* © 1997 Wiley-Liss, Inc.

KEY WORDS: **esophageal carcinoma; lymph node metastasis; AgNOR score**

INTRODUCTION

Nucleolar organizer regions (NORs) are loops of chromosomal DNA that contain clusters of ribosomal RNA genes [1]. They are located in the nucleoli of cells and in the chromosomes 13–15, 21, and 22. Since the NORs are associated with argyrophilic acidic nonhistone proteins, they can be demonstrated by a simple silver staining method as argyrophilic nucleolar organizer regions (AgNORs) in formalin-fixed, paraffin-embedded tissues [2–4]. The number of AgNORs has been reported to correlate with the level of rDNA transcription and the rate of cell proliferation [5]. The number of AgNORs is greater in maligant tumors than in their benign counterparts or in normal tissues [6–10]. Recently, Morita et al. [11] have reported a relationship between AgNORs in biopsy specimens and the malignant potential of esophageal carcinomas. They also found that the AgNOR count was higher in tumors with lymph node metastases than in those with negative nodes. Little is known about AgNOR scores in nodal metastases. In this study, we assessed the relationship between the AgNOR scores of

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the primary tumor and those of lymph node metastases in 54 patients with esophageal carcinoma.

MATERIALS AND METHODS

From 1980 to April 1993, 120 Japanese patients with esophageal carcinoma underwent surgical resection in the Department of Surgery II, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan. All were diagnosed histologically as squamous cell carcinoma. Fifty-four patients with lymph node metastases had received no preoperative irradiation or chemotherapy. In this study, these 54 primary tumors and their 115 lymph node metastases were studied for AgNORs. Evaluations of the extent of disease were made according to the rules of the Japanese Society of Esophageal Disease [12].

Staining Procedure

Tissue was fixed in 10% formalin and processed routinely in paraffin. A 3 μm section cut from the primary tumors and lymph node metastases were mounted on chrome-gel glass slides, dewaxed in xylene, and dehydrated through an alcohol series to deionized and distilled water. The AgNOR staining solution was prepared as described by Egan et al. [13] by adding one volume of 2% gelatin in 1% formic acid to two volumes of 50% aqueous silver nitrate. The tissue sections were incubated with the freshly prepared working solution for 40 minutes in the dark at room temperature (18–20°C).

After thorough rinsing with deionized water to remove nonspecific silver precipitates, the sections were stained with a 2% chloroauric acid solution for 5 minutes to discriminate the AgNORs as clearly staining dots [14]. Sections then were counterstained with Meyer's hematoxylin and dehydrated through graded alcohols to xylene.

Counting Procedure of AgNORs

For each specimen, 100 cancer cells were randomly selected from the primary tumor and from involved portions of the lymph nodes. The number of AgNORs, seen as black dots within the nucleus of each cell, were counted under an oil immersion lens at ×1,000 using an Olympus microscope (BH-2, Olympus, Japan) [15]. We counted all silver-stained strucutures, but when dots were grouped, each cluster was treated as one structure. Counts were performed by one individual (K. Yano) to avoid interindividual variation. Total counts of AgNORs were made for 100 cancer cell nuclei. It was so difficult to find cancer cells in lymphatic vessels that counts of AgNORs were made for 10–30 cancer cell nuclei. The mean number of the AgNORs per nucleus was calculated for each specimen and turned the AgNOR score. In a case with multiple nodal metastases, an average of AgNOR scores for individual involved node was taken as the AgNOR score.

Statistical Methods

Analysis of AgNORs counts for each clinicopathological characteristic and statistical comparison of the AgNORs number among primary tumor, site of lymphatic invasion, and lymph node metastases were carried out using Student's unpaired *t*-test and Mann-Whitney U test, or the Kruskal-Wallis test. Survival curves were calculated by the Kaplan-Meier method. The generalized Wilcoxon test was used for statistical comparison of survival curves.

RESULTS

The clinicopathological findings considered in relation to AgNOR scores of the primary tumors and lymph node metastases are summarized in Table I. There were no statistically significant associations except for sex and venous invasion. Particularly, the AgNOR scores of nodal metastases did not change according to the anatomic level of nodal involvement (n factor).

As shown in Table I, the mean AgNOR score of the 54 primary tumors was 3.9 ± 0.9 (S.D.), whereas that of the 115 nodal metastases was 2.1 ± 0.6. The mean AgNOR score of metastatic lymph nodes was significantly lower than that of the primary tumors ($P = 0.0001$).

The AgNORs of cancer cells at the site of lymphatic vessel invasion in the primary tumor were counted in 30 cases, with the average of these scores being 2.9 ± 0.6 (S.D.), significantly lower than that of the primary tumors ($P = 0.0001$) and significantly higher than that of the metastatic nodes ($P = 0.0001$) (Fig. 1).

Considering the mean AgNORs of the primary tumors and the nodal metastases, we divided them into two groups. Figure 2 shows survival curves of the patients according to the AgNORs score of the primary tumor. The survival of 22 patients with an AgNOR score >4.0 was significantly lower than that of 32 patients with an AgNOR score <4.0 (2-year survival rate, 16% and 49%, 3-year survival rate, 5% and 29%, respectively, $P = 0.0014$).

There was no significant difference in survival between patients with AgNOR scores ≧2 (30 cases) and <2 (24 cases) for the lymph node metastases (Fig. 3).

The AgNOR scores of the lymph nodes were smaller than those of the primary tumor in 53 of 54 cases (98.1%). There was no correlation of AgNOR score between primary tumors and metastatic lymph nodes (Fig. 4).

Our previous report demonstrated that the number of lymph nodes with metastasis correlated well with the prognosis of esophageal cancer. Among the 54 present cases, there were 36 patients with four or fewer lymph nodes showing metastasis and 14 patients with tumor in

TABLE I. Esophageal Carcinoma: Correlation Between Clinicopathological Findings and AgNOR Score

		AgNORs score		AgNors score	
	Cases	Primary tumors	*P* value	Lymph nodes metastases	*P* value
Sex					
Male	47	4.0 ± 0.9[a]	0.01	2.1 ± 0.5	N.S.
Female	7	3.4 ± 0.5		2.0 ± 0.2	
Age (years)					
<60	16	4.0 ± 0.9		2.1 ± 0.4	
60≤ <70	26	3.8 ± 0.9	N.S.	2.0 ± 0.4	N.S.
70≤	12	3.9 ± 0.9		2.3 ± 0.4	
Site of tumor					
Cervical	6	4.3 ± 0.6		2.0 ± 0.4	
Upper thoracic	5	3.8 ± 0.9		2.3 ± 0.5	
Middle thoracic	32	3.9 ± 0.9	N.S.	2.1 ± 0.5	N.S.
Lower thoracic	11	3.7 ± 0.9		2.0 ± 0.3	
Length of tumor					
<5.0	14	3.5 ± 0.7		2.0 ± 0.4	
5.0≤	40	4.0 ± 0.9	N.S.	2.1 ± 0.4	N.S.
Histologic type					
Well	8	3.9 ± 0.9		2.1 ± 0.3	
Moderately	26	4.1 ± 0.9		2.2 ± 0.7	
Poorly	18	3.8 ± 0.8	N.S.	2.0 ± 0.4	N.S.
Others	2	2.9 ± 0.2		2.1 ± 0.2	
Depth of cancer invasion[b]					
<sm	5	3.4 ± 0.5		1.9 ± 0.3	
mp	8	3.8 ± 0.9		2.3 ± 0.6	
a1	11	4.1 ± 0.9	N.S.	2.1 ± 0.4	N.S.
a2	14	3.7 ± 0.8		1.9 ± 0.3	
a3	16	4.3 ± 0.9		2.2 ± 0.4	
Venous invasion					
negative	21	3.6 ± 0.8		2.0 ± 0.3	
positive	33	4.1 ± 0.9	0.05	2.1 ± 0.5	N.S.
Lymphatic invasion					
negative	5	3.3 ± 0.3		1.9 ± 0.4	
positive	49	4.0 ± 0.9	N.S.	2.1 ± 0.4	N.S.
Intraepithelial spread					
negative	22	3.8 ± 0.8		2.1 ± 0.6	
positive	32	4.0 ± 0.9	N.S.	2.1 ± 0.6	N.S.
n factor[c]					
n1	2	3.4 ± 0.9		1.8 ± 0.4	
n2	30	3.8 ± 0.9	N.S.	2.0 ± 0.5	N.S.
n3	12	4.2 ± 0.8		2.3 ± 0.7	
n4	10	3.9 ± 1.1		2.0 ± 0.6	
Total	54	3.9 ± 0.9		2.1 ± 0.4	

[a]Standard deviation for all numbers in column.
[b]sm: submucosa; mp: muscle propria; a1: invasion reaching the adventitia; a2: definite invasion to the adventitia; a3: invasion into the neighboring structures.
[c]N.S. = not significant.

five nodes or more. The number of metastases to lymph nodes could not be determined in four cases. Table II shows AgNOR score of primary tumor and lymph node metastases according to number of metastatic nodes. There were no significant differences.

DISCUSSION

Nucleolar organizer regions (NORs) are chromosomal segments that encode ribosomal RNA (rRNA) and are associated with argyrophilic, acidic, nonhistone proteins. Ribosomal RNA genes ultimately direct ribosome and protein synthesis, and it has been suggested that the number of NORs detected in a cell may reflect proliferative activity [4]. AgNOR counts are dependent on the proliferative status of the cell [16]. A correlation has been observed between AgNOR counts and the mitosis-karyorrhexis index in neuroblastoma [13]. Crocker and Nar [5] reported that a significant difference was found between the numbers of AgNORs in the nuclei of low-grade lymphomas (a mean of 1–1.5 per nucleus) and

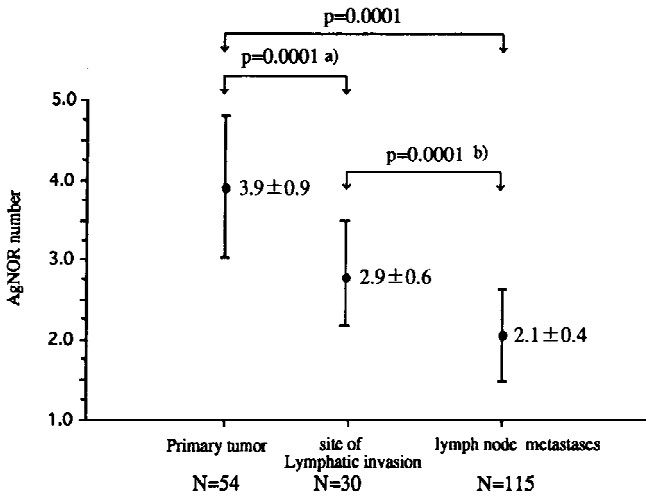

Fig. 1. Comparison of AgNOR scores among primary tumors, site of lymphatic invasion and metastatic lymph nodes. (a) There was a significant difference between the AgNOR score of the primary tumor and that of the cancer cell at the site of lymphatic vessels (P = 0.0001). (b) There was a significant difference between the AgNOR score of the lymph node metastases and the cancer cell at the site of lymphatic vessels (P = 0.0001).

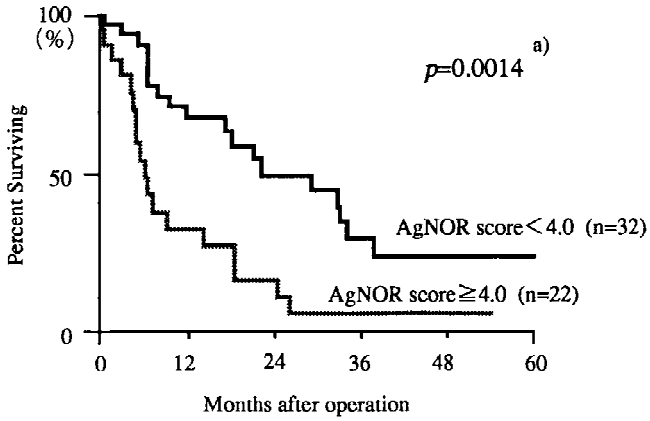

Fig. 2. Survival curves of patients with esophageal carcinoma according to AgNOR score of the primary tumor. (a) There was a significant difference in survival between patients with AgNOR score ≧ and <4 for the primary tumor (P = 0.0014).

those of high-grade lymphomas (a mean of 4.4–6.8 per nucleus). Some reports have proposed that the AgNORs score mirrors proliferative activity in tumors such as adenoid cystic carcinoma lung cancer, hepatoma, and colonic cancer [6,8,17–21].

In esophageal carcinoma, Morita et al. [11] have reported that the AgNOR count was higher in more advanced stages of esophageal carcinoma and that the survival of the patients with a high AgNOR number was significantly poorer than that of patients with a low AgNOR number. Our present series of advanced esophageal carcinoma also indicated similar results on survivals according to the AgNOR scores of the primary tumor (Fig. 2). Thus the AgNOR number of the primary lesion is a

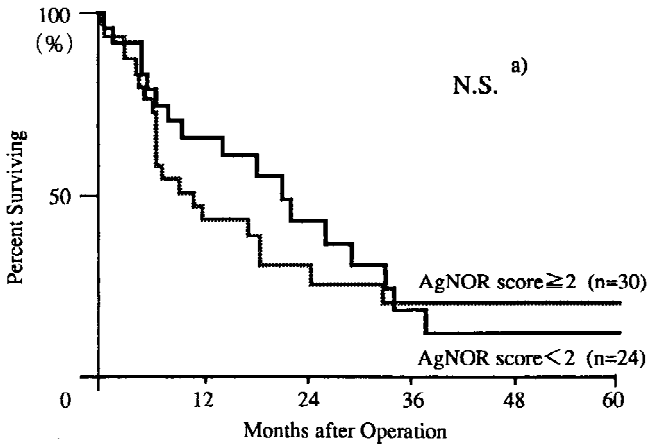

Fig. 3. Survival curves of patients with esophageal carcinoma according to AgNOR score of lymph node metastases. (a) There was no significant difference in survival between patients with AgNOR score ≧2 and <2 for the lymph node metastases. N.S. = not significant.

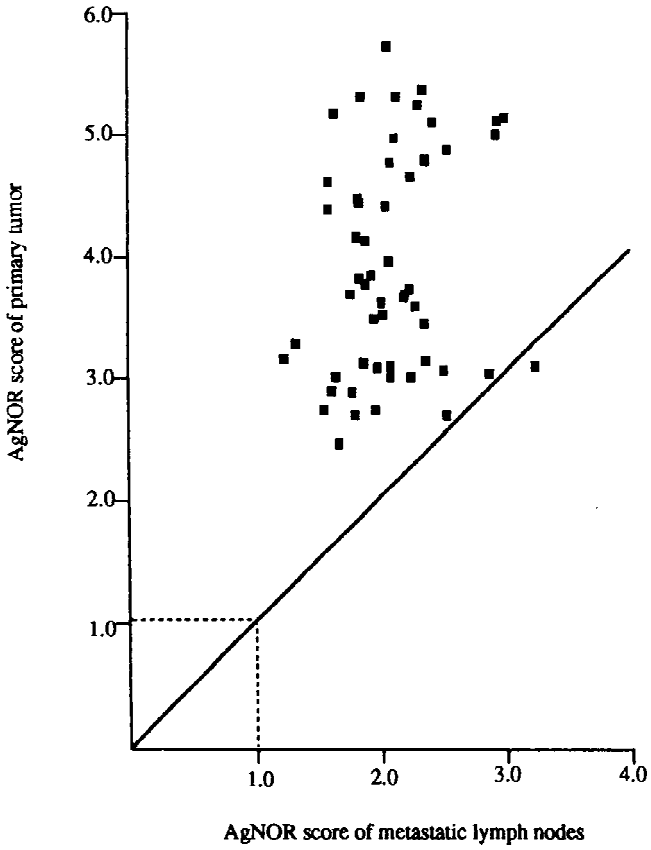

Fig. 4. Correlation of AgNOR score between primary tumor and metastatic lymph nodes.

good indicator of malignant potential in esophageal carcinoma.

Although aggressive surgical treatment and a number of new adjuvant therapies have been implemented [22,23], the prognosis of patients with esophageal carcinoma remains poor. Five-year survival rates as reported

TABLE II. Esophagael Carcinoma: AgNOR Score of Primary Tumor and Lymph Node Metastasis According to Number of Metastatic Nodes

Number of metastatic lymph nodes	AgNOR score	
	Primary tumors	Lymph nodes
≦4 (36 cases)	3.8 ± 0.9[a]] N.S.	2.1 ± 0.4]N.S.
5≧ (14 cases)	4.2 ± 0.9	2.1 ± 0.5

[a]Standard deviation.
N.S. = not significant.

in the world literature are between 8–20% [24,25]. The poor prognosis of such patients is partly explained by the finding that most esophageal carcinomas involve the adjacent structures and regional lymph nodes at the time of operation [26,27]. It has been reported that the prognosis of patients with nodal metastases was poorer than that of patients with negative lymph nodes [28,29]. In our present study, the 5-year survival rate of the 54 patients with lymph node metastasis was 17% compared with 53% of those without lymph node metastasis (data not shown). Morita et al. [11] reported that AgNOR score in primary tumors was higher in the n(+) cases than in the n(−) cases. However, there have been no definite studies concerning the proliferative activity in metastatic lymph nodes of esophageal carcinoma by AgNOR analysis.

Our study was conducted to compare AgNOR scores among primary tumor, cancer cells seen in lymphatic vessels, and lymph node metastases. The AgNOR scores were highest in primary tumors, intermediate in foci of lymphatic vessel invasion site, and lowest in lymph node metastases (Fig. 1). Our previous report [29] indicated that prognosis of patients with five or more lymph nodes metastases was poorer than that of those with 1–4 node metastases. Therefore, AgNOR scores of primary tumor and lymph node metastases were assessed according to number of lymph nodes with metastasis. As shown in Table II, AgNOR scores in the metastatic lesions were low and comparable in the both groups. Thus cell proliferation as reflected by AgNOR scores may have been suppressed at lymphatic invasion sites and suppressed further in lymph nodes.

Since previous studies reported that high AgNOR score represented high proliferative activity [9–11], the decrease of the AgNOR scores of the metastatic lymph nodes was contrary to our expectation and biological significance of which was difficult to explain. In gastric carcinoma, Baba et al. [30] reported that malignant potential of the metastatic nodes, measured by DNA ploidy, were reduced compared with that of the primary tumors and suggested that reduction in DNA content during the metastatic process could be due to selection of subpopulations with a high potential to metastasize [30]. If the lower AgNOR score of metastatic lesions can be explained by selection of subpopulation, microenvironment in the lymphatic system may be preferable for the tumor cells with lower proliferative activity. As reported previously, monocyte-macrophage series of cells resident in the area of the primary tumor, in lymph nodes, and in peripheral blood exert antitumor effects evident as a suppression of DNA synthesis of target tumor cells [31,33]. The low AgNOR scores of lymph node metastases may reflect relatively high antitumor activity of macrophages in involved lymph nodes compared with that of macrophages near the primary tumor, a possibility awaiting further elucidation.

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